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Effect of Jiaji electroacupuncture in transected rat spinal cord

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We evaluated the effect of Jiaji electroacupuncture on cell proliferation and the expression of markers of endogenous neural stem cell activation after complete spinal cord transection. Female Wistar rats were assigned to 4 groups (n = 24 each): a sham-operated group, a control group, a Jiaji electroacupuncture group, and a Jiaji electroacupuncture preconditioning group. Motor function was significantly improved in the acupuncture groups compared to the control group at 7 and 14 d. Numbers of bromodeoxyuridine (BrdU)-, nestin-, and glial fibrillary acidic protein (GFAP)-positive cells were significantly greater in the acupuncture groups than in the controls at each time point. Expression of nestin and GFAP mRNA was significantly higher in the acupuncture groups than in the controls at each time point. Thus, Jiaji electroacupuncture and preconditioning may promote the proliferation of endogenous neural stem cells after spinal cord transection.

Jiaji electroacupuncture, spinal transection, rat, endogenous neural stem cells

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Spinal cord injury can cause disability or death. It has long been believed that the adult mammalian central nervous system is incapable of regeneration or repair and that damage results in permanent disability. However, many recent studies have identified and characterized neural stem cells in adult mammals, including human, that show some central nervous system renewal ability [1]. Neural stem cells are normally dormant; however, they can be induced by stimuli such as damage or ischemia to proliferate and migrate to sites of damage to replace necrotic neurons, repair lesions, and restore neural function. This brings new hope for successful treatment of spinal cord injuries. At present, there are 2 treatment approaches for spinal cord injury using neural stem cells: transplantation of exogenous neural stem cells and activation of endogenous neural stem cells. Although much progress has been made with respect to the former, technical issues such as immunologic rejection and ethical issues regarding the use of stem cells remain [2]. Therefore, activation of endogenous neural stem cells may be more appropriate for clinical application.

In 1920, it was first reported that electric fields play a role in nerve growth and reduction of injured nerve degeneration [3]. Electrical stimulation can promote the proliferation and differentiation of endogenous neural stem cells and has been reported to enhance the reparative ability of nervous tissue [4,5]. Acusector and electric field treatments have been used for spinal cord injuries in both experimental animals and clinical practice [6–9]. Jiaji, also termed “Xieji” or “Xiaji,” refers to acupuncture points (acupoints) located on either side of the vertebral column. References to these points were found in the early Chinese medical literature (Huangdi Neijing). Cheng [10] confirmed the locations of these acupoints (termed Hua Tuo Jiaji acupoints) bilaterally, 15 mm below the spinal processes from T1 to L5 and totaling 17 pairs. Jiaji acupuncture entails the use of 4 needles: 2 above the level of damage and 2 below. Jiaji electroacupuncture involves the application of an electric current to the needles [11]. A pulsed electric field reportedly promotes vascularization and regeneration of nervous tissue [12], and electroacupuncture has been shown to promote functional

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recovery after spinal cord injury [13]. This method improves blood circulation and relieves edema after spinal cord injury [14], and it can protect and promote axonal regeneration [15,16]. The electromagnetic field effects of electroacupuncture promote neuronal polarization, enzymatic activity [17], and gene expression [18,19].

We were interested in determining whether Jiaji electroacupuncture can activate neural stem cells in the context of the injured spinal cord. We were interested specifically in the Jiaji point because of its position relative to the spinal cord. Therefore, the aim of the present study was to investigate the effect of Jiaji electroacupuncture on markers of endogenous neural stem cell activation, including bromodeoxyuridine (BrdU), nestin, and glial fibrillary acidic protein (GFAP), after complete spinal cord transection in rats.

1 Materials and methods

1.1 Animals and study design

The animal treatment protocol was approved by the Review Board of Heilongjiang University of Chinese Medicine (Harbin, China). Ninety-six adult (11–12 weeks of age; 200–220 g) female Wistar rats (Animal Experiment Center, Heilongjiang University of Traditional Chinese Medicine) were randomly assigned to 1 of 4 groups: a sham-operated group in which the spinal cord was exposed but not transected; a control group in which the spinal cord was completely transected at T10 but not treated; a Jiaji electroacupuncture group in which rats were treated for 3, 7, or 14 d after transection; and a Jiaji electroacupuncture preconditioning group in which rats were treated for 1 week before transection in addition to treatment after transection. Rats in each group were killed at 3, 7, and 14 d after transection (n = 8 per time point in each group). Spinal cord tissues from 4 rats at each time point were assessed by immunohistochemistry, and 4 were assessed by real-time quantitative polymerase chain reaction (PCR). We also assessed neural conduction from L2 to T8 in each group.

1.2 Spinal cord transection

For spinal cord transection, rats were anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneal injection) and fixed in a prone position. The back skin was disinfected, the skin and subcutaneous tissue were cut, and the spine was exposed at the level of T8 to T10. The neural scute was cut with surgical forceps, and the spinal cord was exposed at the level of T9 to T11. The spinal cord was completely transected at the level of T10 [20] with microscissors [21,22] and then covered with a gelatin sponge. The wound was stitched, and 1.6×10^5 U penicillin was infused intraperitoneally once a day for 5 d. The bladder was stimulated by pressure until recovery of micturition, and 0.1 mg/kg neostigmine was administered to promote gastrointestinal peristalsis. After recovery, food and water were supplied ad libitum.

1.3 Jiaji electroacupuncture and assessment of motor function

The Jiaji method was applied according to Lin [23] and Zhang et al. [24]. The acupuncture position was 3 to 4 mm from the clearance of the 2 adjacent processus spinosus vertebrae. The 2 upper needles were placed beside the clearance of the 2 processus spinosus vertebrae above the level of transection. Two additional needles were placed beside the clearance of the 2 processus spinosus vertebrae below the level of transection. The 25-mm Hua Tuo needles were placed vertically at a depth of 4 to 5 mm, with the tip of each needle touching the lamina. An electric acupuncture stimulator (KWD-808 II; Great Wall, Jiangsu, China) was used to apply a pulse current to the needle handle, with the positive pole at the upper needle handle and the negative pole at the lower needle handle. A condensation wave was applied at a frequency of 100 Hz, and the output current was set to activate mild twitching of the back muscles. Rats in the Jiaji electroacupuncture group were treated for 15 min at 30 min, 4 h, and 8 h after transection and then once a day for 3, 7, or 14 d. Rats in the Jiaji electroacupuncture preconditioning group were treated for 15 min once a day for 1 week before transection. After transection, the treatment was the same as that for the electroacupuncture group. Motor function was assessed after spinal cord transection according to the Basso, Beattie, and Bresnahan (BBB) open-field locomotor scale [25]. Two experienced observers performed scoring at 3, 7, and 14 d after transection, and the scores were averaged.

1.4 Immunohistochemistry

Rats were administered an intraperitoneal injection of 1% of BrdU (150 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) in physiologic saline 3 d before they were killed by injection with 10% chloral hydrate (400 mg/kg) and perfused with 4% paraformaldehyde. The spinal cord surrounding the level of transection was removed and placed in 4% paraformaldehyde for 24 h and then embedded in paraffin. For immunohistochemistry, 2-μm-thick tissue sections were dewaxed, and antigen retrieval was performed by steam treatment. Sections were incubated in 3% hydrogen peroxide for 10 min and then incubated overnight with monoclonal BrdU antibody (1∶200; NeoMarkers, Fremont, CA, USA), polyclonal nestin antibody (1∶400; Abcam, Cambridge, UK), or GFAP antibody (1∶400; Wuhan Boster Biological Technology, Wuhan, China) at 4°C and then incubated with reagents of a PV6001/6002 2-step immunohistochemistry kit (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 30 min at room temperature. 3,3′-Diaminobenzidine developer was then added dropwise

while observing the development of a yellow-brown color under a microscope (Nikon, Tokyo, Japan) for 10 to 30 min. Sections were counterstained with hematoxylin. Positive cells were counted for 5 randomly selected microscopic fields (400×) per section.

1.5 Real-time PCR

For RNA isolation, rats were killed by decapitation, and the spinal cord surrounding the transection was rapidly removed and stored at −80°C until use. Total RNA was isolated with RNAiso Plus reagent (Takara Bio, Shiga, Japan). Reverse transcription (RT) was performed in a volume of 10 μL containing 5× PrimeScript Buffer (2 μL), PrimeScript RT Enzyme Mix (0.5 μL), Oligo dT Primer (0.5 μL), random 6 mers (0.5 μL), total RNA (4 μL), and RNase-free H_2O (2.5 μL) (all from Takara Bio). The reaction was performed for 15 min at 37°C followed by 5 s at 85°C. Amplification products were stored at −80°C until use in PCR. Primer sequences for real-time fluorescence quantitative PCR were as follows (Takara Bio): glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (143 bp), 5′-GGCACAGTCAAGGCTGAGAATG-3′ and 5′-ATGGTGGTGAAGACGCCAGTA-3′; GFAP (163 bp), 5′-AGTGGCCACCAGTAACATGCAA-3′ and 5′-GGACTCAAGGTCGCAGGTCAA-3′; and nestin (101 bp), 5′-CAGCAACTGGCACACCTCAAG-3′ and 5′-CCTCGTCCAGGTGTCTGCAA-3′. Relative GFAP and nestin mRNA expression was calculated according to the threshold cycle method with the use of GAPDH as an internal control [26]. PCR was performed in a total volume of 25 μL, including RT-PCR mix and 0.5 μL of each primer. The PCR condition was as follows: 30 s at 95°C followed by 40 cycles of 5 s at 95°C for denaturation and 30 s at 60°C for annealing and elongation. Dissociation curve analysis was performed after PCR. Agarose gel electrophoresis was performed to verify amplification. For assessment of amplification efficiency, a standard curve was created for each PCR target with diluted cDNA (20, 10, 5, 2.5, and 1.25 ng) plotted versus corresponding Ct; amplification efficiency was calculated according to $E = 10^{-S} - 1$.

1.6 Assessment of neural conduction

To assess neural conduction, rats were anesthetized with an abdominal injection of 10% chloral hydrate (400 mg/kg) and placed in the prone position. Incisions were made to expose the skull, L2 and T8 of the spinal cord, and the sciatic nerve of the left hind limb. A Danmark Dantec Keypoint electromyography/evoked potential (EP) system (Skovlunde, Denmark) was used to assess EPs of somesthetic sensation and conduction speed. Electrodes were 15-mm Hua Tuo filiform needles. The recording electrode for the spinal cord was fixed to the intraspinous ligament between L1 and L2. The recording electrode for the cortex was fixed to the intraspinous ligament between T7 and T8.

Reference electrodes were fixed 1 to 2 cm from recording electrodes. The stimulation electrode was fixed to the caput fibulae. An appropriate level of stimulation was determined to be that which generated small motions of the ankle joint. Stimulation was set at 0.4 to 0.8 mA, the stimulation period was 0.2 ms, frequency was 30 Hz, superposition time was 200 times, scanning speed was 3 ms/D, and sensitivity was 10 μV/D. The stimulation point on the body surface was fixed to the other caput fibulae. We then assessed the conduction time from the recording stimulation point to the spinous process of L1 to L2 and T7 to T8 and the spinal cord conduction velocity (SCCV) from L2 to T8.

1.7 Statistical analysis

Data are presented as mean ± standard deviation for normally distributed continuous variables and were tested by 1-way analysis of variance. Tukey's test or Dunnett's test was then utilized for post hoc analysis of continuous variables with or without equal variance between groups. The BBB score is presented as the median (interquartile range) because of non-normal distribution. The difference in BBB score among groups was verified by the Kruskal-Wallis test, and the Mann-Whitney *U* test was applied for post hoc analysis of BBB scores. A 2-sided $P < 0.05$ was considered statistically significant, and the significance level was adjusted to 0.005 (= 0.05/number of post hoc tests) when the Mann-Whitney *U* test was implemented. Statistical analyses were performed with SPSS statistical software (v. 15.0; SPSS Inc., Chicago, IL, USA).

2 Results

2.1 Motor function

BBB scores for the 4 groups of rats are listed in Table 1. At day 3 after transection, a significant ($P < 0.001$) difference between the sham group and the other 3 groups was observed, as expected. However, motor function scores were significantly ($P < 0.001$) higher in the acupuncture treatment groups (electroacupuncture and preconditioning) than in the control group (transection but no acupuncture treatment) at days 7 and 14 after transection.

2.2 Immunohistochemistry and PCR results

Immunohistochemical staining for BrdU, nestin, and GFAP is shown in Figures 1–3, and quantification of immunopositivity for these markers among the 4 groups is summarized in Table 2. The numbers of BrdU-, nestin-, and GFAP-immunopositive cells were significantly ($P < 0.001$) greater in the acupuncture treatment groups than in the control group at each time point. In addition, the effect was greater in the preconditioning Jiaji electroacupuncture group than in the group that received electroacupuncture after transection

Table 1 Motor function of different groups 3, 7 and 14 d after spinal cord transection as assessed by Basso, Beattie and Bresnahan scores[a)]

	Sham (n = 8/time point)	Control (n = 8/time point)	Electroacupuncture (n = 8/time point)	Preconditioning (n = 8/time point)	P value
Day 3	21.0 (21.0, 21.0)[a]	0 (0, 0)[b]	0 (0, 0)[b]	0 (0, 0)[b]	<0.001*
Day 7	21.0 (21.0, 21.0)[a]	1.0 (0, 1.0)[b]	2.0 (1.5, 2.0)[c]	4.0 (3.5, 4.5)[d]	<0.001*
Day 14	21.0 (21.0, 21.0)[a]	4.0 (3.0, 4.5)[b]	6.0 (5.5, 6.0)[c]	8.5 (7.5, 9.5)[d]	<0.001*

a) Data are presented as median (interquartile range) and were tested by Kruskal-Wallis test; Mann-Whitney U test with adjusted α' (= 0.05/10 = 0.005) was implemented for post-hoc analysis. *, Significantly different among the 4 groups. Different letters (a, b, c, d) indicate significant ($P < 0.005$) differences between groups.

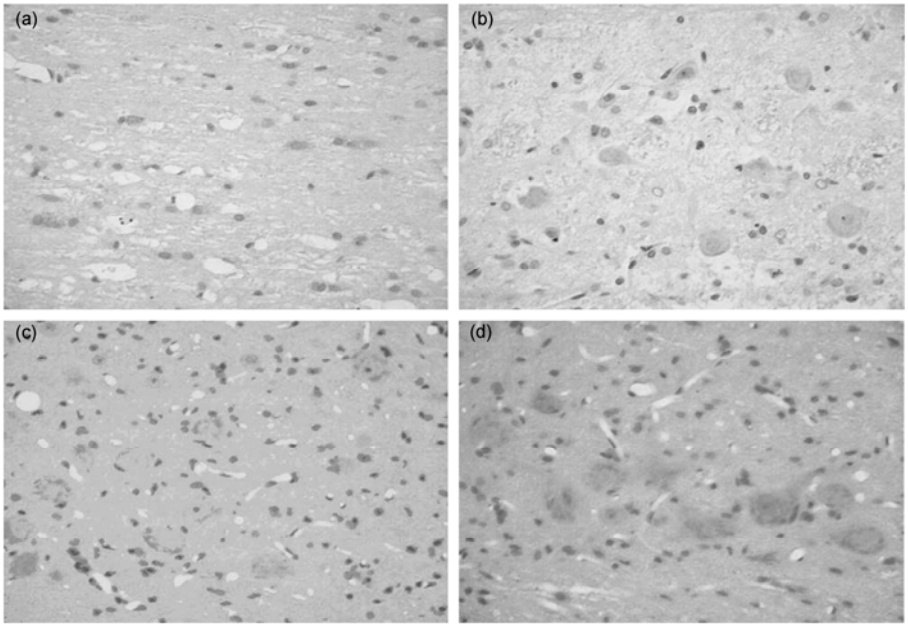

Figure 1 Representative images of nestin immunostaining in spinal cord tissue from sham-treated rats (a), control rats (b), rats receiving electroacupuncture preconditioning treatment (c), and rats receiving electroacupuncture treatment for 1 week after spinal cord transection (d).

alone. mRNA expression for these neural stem cell markers is summarized in Table 3. The expression of nestin and GFAP mRNA was significantly ($P < 0.001$) greater in the treatment groups than in the control group at each time point.

2.3 Neural conduction

Table 4 shows the numbers of rats in each group with detectable conduction from L2 to T8 at different times after sectioning. Conduction speed in the sham group was faster than that in the electroacupuncture or preconditioning group at each time point after sectioning. On the 14th day after sectioning, conduction speed in the electroacupuncture group (12.13 m/s; n = 4) and the preconditioning group (25.04 m/s; n = 7) was faster than that in the control group (3.01 m/s; n = 3). In addition, conduction speed in the electroacupuncture and preconditioning groups on the 14th day were faster than those at the 7th day (3.08 m/s in the electroacupuncture group (n = 3) vs. 12.13 m/s in the preconditioning group (n = 4)).

3 Discussion

The aim of the present study was to assess the effect of Jiaji electroacupuncture on cell proliferation and the expression of nestin and GFAP, as markers of neural stem cells, in transected rat spinal cord. Results showed that Jiaji electroacupuncture, applied either after transection or both before and after transection, increased cell proliferation in the spinal cord as assessed by BrdU staining and cell quantification and increased the number of cells positive for nestin and GFAP immunostaining compared with the control group.

We assessed 3 markers of neural stem cells in this study. BrdU intercalates into DNA and is used as a marker of cell division and proliferation. Nestin, also termed nidogen, is an intermediate filament protein expressed in neuroepithelial precursor cells and adult pluripotent stem cells [27]. GFAP is commonly used as a marker of glial response to injury, and astrocytes can differentiate along multiple lines and exert various effects in response to spinal cord injury [28,29]. In fact, Lang et al. reported that astrocytes can revert

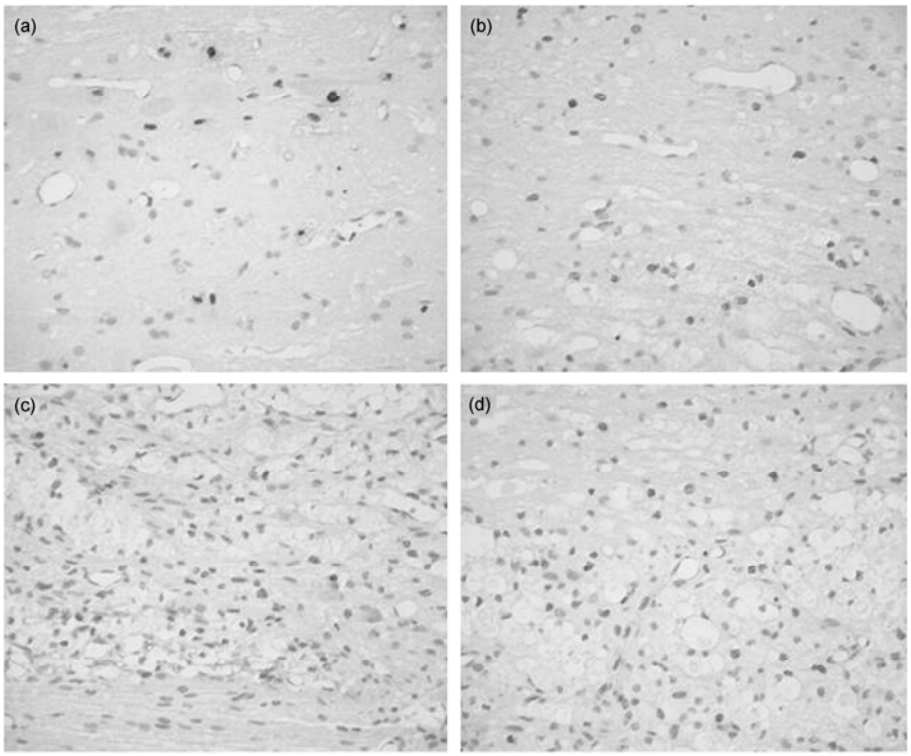

Figure 2 Representative images of bromodeoxyuridine (BrdU) immunostaining in spinal cord tissue from sham-treated rats (a), control rats (b), rats receiving electroacupuncture preconditioning treatment (c), and rats receiving electroacupuncture treatment for 1 week after spinal cord transection (d).

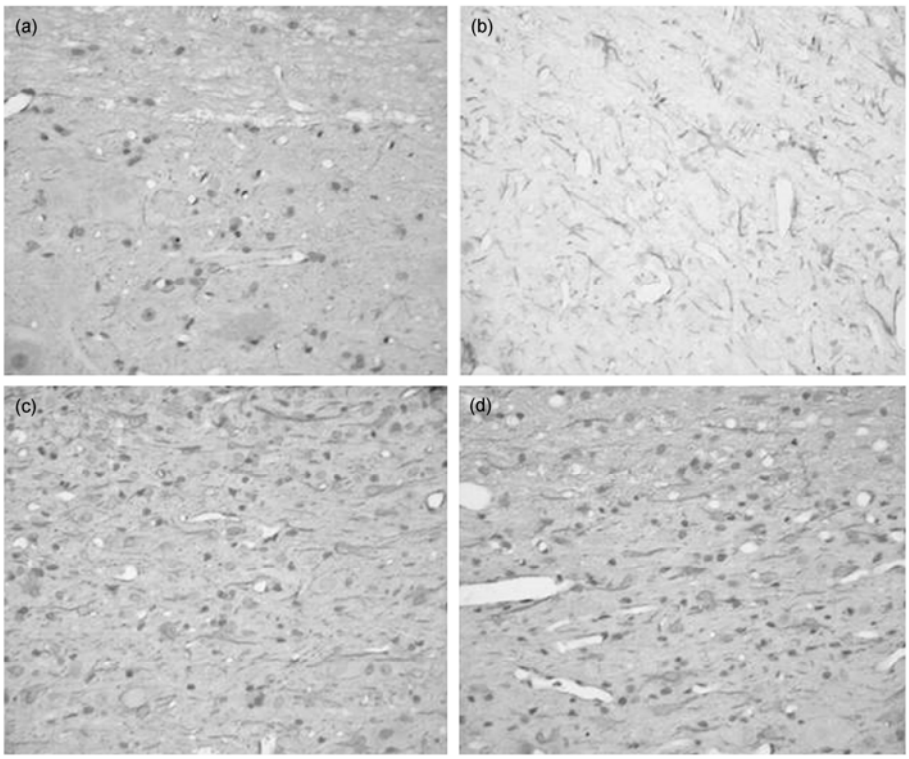

Figure 3 Representative images of glial fibrillary acidic protein (GFAP) immunostaining in spinal cord tissue from sham-treated rats (a), control rats (b), rats receiving electroacupuncture preconditioning treatment (c), and rats receiving electroacupuncture treatment for 1 week after spinal cord transection (d).

Table 2 Neural stem cell marker immunopositivity among groups (cell number per square mm)[a)]

	Sham (n = 4/time point)	Control (n = 4/time point)	Electroacupuncture (n = 4/time point)	Preconditioning (n = 4/time point)	P value
Day 3					
Nestin	4.7±1.9[a]	38.4±5.4[b]	86.3±2.3[c]	111.3±3.7[d]	<0.001*
BrdU	60.9±6.5[a]	263.8±9.8[b]	590.9±18.7[c]	860.9±22.0[d]	<0.001*
GFAP	34.1±3.4[a]	73.4±4.7[b]	183.4±4.1[c]	274.7±19.6[d]	<0.001*
Day 7					
Nestin	3.4±1.2[a]	67.8±5.3[b]	123.4±5.1[c]	170.0±5.9[d]	<0.001*
BrdU	60.9±8.3[a]	416.6±20.2[b]	890.9±39.5[c]	1508.1±26.0[d]	<0.001*
GFAP	30.6±5.3[a]	129.7±11.0[b]	276.3±22.9[c]	442.8±8.3[d]	<0.001*
Day 14					
Nestin	5.0±1.0[a]	30.9±3.7[b]	74.7±2.1[c]	104.4±3.9[d]	<0.001*
BrdU	55.6±8.6[a]	99.1±4.7[b]	439.1±12.2[c]	834.7±19.2[d]	<0.001*
GFAP	32.2±4.5[a]	48.8±6.5[b]	172.5±8.4[c]	231.6±13.3[d]	<0.001*

a) Data are presented as mean ± standard deviation (SD) number of cells, and differences were tested by 1-way analysis of variance (ANOVA). Tukey or Dunnett test was used for post-hoc analysis. *, Significantly different among the 4 groups. Different letters (a, b, c, d) indicate significant ($P < 0.05$) differences between groups. BrdU, bromodeoxyuridine; GFAP, glial fibrillary acidic protein.

Table 3 mRNA expression for neural stem cell markers among groups[a)]

	Sham (n = 4/time point)	Control (n = 4/time point)	Electroacupuncture (n = 4/time point)	Preconditioning (n = 4/time point)	P value
Day 3					
GAPDH	16.4 ± 0.7	17.1 ± 0.5	17.0 ± 0.4	17.1 ± 0.5	0.249
GFAP	19.8 ± 0.9[a]	17.3 ± 1.0[b]	14.5 ± 0.5[c]	13.9 ± 0.4[c]	<0.001*
Nestin	27.4 ± 0.6[a]	23.7 ± 0.7[b]	22.2 ± 0.7[c]	21.1 ± 0.3[c]	<0.001*
Day 7					
GAPDH	17.0 ± 0.4	17.7 ± 0.2	17.7 ± 0.7	17.6 ± 0.3	0.108
GFAP	20.0 ± 0.7[a]	16.9 ± 0.6[b]	14.7 ± 0.5[c]	12.2 ± 0.6[d]	<0.001*
Nestin	27.0 ± 0.9[a]	24.8 ± 0.5[b]	22.0 ± 0.4[c]	19.8 ± 0.4[d]	<0.001*
Day 14					
GAPDH	17.3 ± 0.7	17.9 ± 0.5	17.5 ± 0.5	17.5 ± 0.6	0.567
GFAP	19.3 ± 0.6[a]	17.4 ± 0.4[b]	14.7 ± 0.6[c]	13.8 ± 0.6[c]	<0.001*
Nestin	27.5 ± 0.8[a]	24.6 ± 0.5[b]	22.0 ± 0.5[c]	20.8 ± 0.6[c]	<0.001*

a) Data are presented as mean ± standard deviation (SD) of cycle numbers (smaller numbers indicate higher amounts) and were tested by 1-way analysis of variance (ANOVA). Tukey or Dunnett test was used for post-hoc analysis. *, Significantly different among the 4 groups. Different letters (a, b, c, d) indicate significant ($P < 0.05$) differences between groups. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein.

Table 4 Numbers of rats with detectable L2–T8 conduction

	Sham (n = 8/time point)	Control (n = 8/time point)	Electroacupuncture (n = 8/time point)	Preconditioning (n = 8/time point)
Day 3	8	0	1	1
Day 7	8	1	3	4
Day 14	8	3	4	7

to neural stem cells after spinal cord injury in rats [30]. Therefore, we believe that the combination of these 3 markers is indicative of neural stem cell activation.

Results of motor function assessments were mirrored by neural conduction experiments, showing a lack of spinal cord EPs after transection followed by partial recovery within 7 to 14 d. This improvement was enhanced by Jiaji electroacupuncture, and particularly by preconditioning. We did not perform a quantitative comparison of wave amplitude height, but visual assessment showed no marked differences between groups. EP conduction in the spinal cord was shorter and spinal cord conduction velocity was elevated in the electroacupuncture groups (more so in the preconditioning group) compared with the control group at 7 and 14 d after transection.

Studies have shown that acupuncture preconditioning can

exert favorable protective effects on hypoxia and ischemia in the heart, brain, gastrointestinal tract, and spinal cord, and it has been suggested that preconditioning induces a protective effect via generation of a trigger factor [31]. Our present results showed a greater effect in the Jiaji electroacupuncture preconditioning group than in the postinjury electroacupuncture treatment group. Thus, preconditioning may also promote endogenous neural stem cell activation. Future studies will be required to confirm the present findings and to further elucidate the mechanisms underlying the effects of Jiaji electroacupuncture on endogenous neural stem cells.

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